DETECTING GLUTARALDEHYDE IN CHEMICALLY HARDENED PROTEINS

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INTRODUCTION

The Explanatory Notes to heading 39.13 of the Harmonized Commodity Description and Coding System define hardened proteins as "nitrogenous compounds of very high molecular weight of vegetable or animal origin . . . which have been chemically processed to harden them" [1]. Unhardened proteins are covered by Chapter 35, while the edible preparations of unhardened and heat hardened proteins are classified in heading 21.06.

Food grade protein sheets and casings are used in the meat processing industry (e.g., for wrapping ham or as artificial guts for sausages). Depending on the processing requirements, such as the type of meat or the speed of packing, they can be either chemically hardened or heat hardened [2]. The heat hardened proteins are not chemically hardened proteins.

From the classification perspective, it is essential to be able to distinguish between chemically hardened and unhardened proteins. Physical properties, such as the tensile strength could be tested, but the analysis would require difficult to obtain standards. As an alternative approach, this paper describes a simple method for detecting traces of glutaraldehyde which is frequently used as a hardening agent.

EXPERIMENTAL

1 PRINCIPLE

Parts per million of glutaraldehyde in the pyrolysate of hardened protein are derivatized using a fluorinated hydroxylamine reagent. The resulting oxime derivative is detected by Gas Chromatography - Mass Spectrometry (GC-MS).

2 APPARATUS

2.1 GC-MS

A Carlo-Erba GC instrument interfaced with a High Resolution Kratos MS25RF Mass Spectrometry Detector was used in all qualitative studies.

The following parameters and conditions were used:

column:	DB-5, 20 m capillary column, 0.18 mm I.D., 0.18 µm film thickness
carrier gas:	helium with a head pressure of 140 kPa
detector:	MS (Electron Multiplier) set to scan at 0.6 sec. per decade from mass 29 to 649 amu
injector:	250°C
oven conditions:	80°C initial temperature held for 1 min., ramped at 25°C/min to 300°C
interface to MS:	290°C
ionization mode:	EI, 70 eV

2.2 Bunsen burner

2.3 Heating plate equipped with a thermostat

2.4 Forced air oven

2.5 GLASSWARE

2.5.1 Round bottom flask with a side arm, 50 ml

2.5.2 Round bottom flask, 50 ml

2.5.3 Separatory funnel, 50 ml

2.5.4 Test tubes

2.5.5 **Beakers, 50 ml**

2.5.6 **Pasteur pipettes**

2.5.7 **Vials with septa and screw caps, 7 and 2 ml**

3 REAGENTS

3.1 Glutaraldehyde, 25 wt.% solution in water

The reagent was obtained from Fisher Scientific.

3.2 Pentafluorobenzylhydroxylamine, PFBHA

The reagent was obtained from Aldrich Chemical Co. A solution containing 40 milligrams of PFBHA in 10 ml of pyridine was prepared and stored refrigerated at 4°C.

3.4 Ethyl acetate, H.P.L.C. grade

3.5 Toluene, H.P.L.C. grade

3.6 Deionized water

Obtained using Solution 2000 Type I Reagent Grade Water Purification System.

3.7 Hydrochloric acid

Concentrated HCl and 2 M HCl are used.

3.8 Sodium sulphate, anhydrous

3.9 Protein casings

The glutaraldehyde hardened and heat hardened casings were obtained from Nitta Casings Inc., Southside Ave., Somerville, NJ, 08878.

4 PROCEDURE

4.1 Pyrolysing the protein

Approximately 2 grams of a protein sample is wetted with concentrated hydrochloric acid and placed into a round bottom flask equipped with a side arm. The flask is capped with a plastic stopper (by Pyrex) having a Teflon liner and the side arm is inserted into a test tube which is slightly longer than the side arm. The test tube is inserted into a 50 ml flask filled with water. The flask with the protein sample is heated with the Bunsen burner using a swinging motion, until approximately 2 ml of pyrolysate is collected.

4.2 Washing with toluene

The pyrolysate obtained in step 4.1 is diluted with deionized water to approximately 5 ml and is transferred into a separatory funnel. The resulting solution is washed twice with 10 ml of toluene to remove hydrocarbon impurities.

4.3 Derivatizing with PFBHA

Approximately 2 ml of washed pyrolysate is placed into a 7-ml screw cap vial and approximately 0.4 ml of the pyridine solution of PFBHA (3.2) is added. The vial is sealed and heated on a heating plate at 60°C for 30 minutes.

4.4 Extracting with ethyl acetate

After cooling to room temperature, the derivatized sample is directly extracted from the 7-ml vial with two 2 ml portions of ethyl acetate. The aqueous layer is discarded.

4.5 Washing with 2 M hydrochloric acid

The two ethyl acetate extracts (top layers from step 4.4) are combined and washed with 2 ml of 2 M HCl to remove the excess of PFBHA. The washing is performed directly in the 7-ml vial.

4.6 Drying with anhydrous sodium sulphate

The top layer from the washing step (4.5) is dried by passing it through a Pasteur pipette filled with anhydrous sodium sulphate. The solution is collected into the 50-ml beaker.

4.7 Concentrating the solution

The ethyl acetate is evaporated from the 50-ml beaker in a forced air oven at 115°C for approximately 15 minutes. After cooling, the content of the beaker is rinsed with approximately 0.2 ml of ethyl acetate and the resulting solution is transferred to a 2-ml vial for the subsequent GC-MS analysis.

4.8 Analysing by GC-MS

The sample is analysed using the conditions specified in 2.1

4.9 Spiking with glutaraldehyde standard

It is necessary to confirm the identity of the detected glutaraldehyde derivative. For this purpose, a solution of 1 ppm glutaraldehyde prepared from a commercial standard is derivatized following steps 4.3 through 4.8.

The derivatized protein pyrolysate is enriched with the standard described above in order to match the retention times and the corresponding mass spectra.

RESULTS AND DISCUSSION

The concept of detecting derivatized glutaraldehyde is not unique in environmental and biochemical analysis, for example, 2,4-dinitrophenylhydrazine (DNPH) **[3]** and 1-methyl-1-(2,4-dinitrophenyl)hydrazine (MDNPH) **[4]** convert aldehydes and ketones to the corresponding hydrazones which are subsequently analysed by HPLC. Similarly, pentafluorobenzylhydroxylamine (PFBHA) affords oxime derivatives detectable by GC-MS **[5]**.

PFBHA has been selected as the derivatizing agent in this study as it has already been proven successful in a similar

industrial analysis [2] and because of the high sensitivity of detection [5]. Unfortunately, strongly interfering peaks from hydrocarbon oil prevented detection of glutaraldehyde O-[(pentafluorophenyl)methyl] oxime in the early experiments (Figure 1). This hydrocarbon impurity was evidently in the form of a non-extractable emulsion, as it remained unchanged, even after washing with various solvents (e.g., pentane, toluene and chloroform). According to the manufacturer, the oil is routinely sprayed on hardened proteins to prevent the loss of moisture [2].

The samples were pyrolysed in order to avoid interference from the oil. To promote disintegration, the protein was acidified by immersing it in concentrated hydrochloric acid. The pyrolysis was performed in a very simple device which can be quickly assembled in any chemical laboratory. The device consisted of a suitably sized round bottom flask equipped with a side arm. The side arm was inserted into a test tube, which in turn was placed inside another flask. This flask was filled with water to condense the pyrolysate.

The chromatograms of the derivatized pyrolysates were much simpler compared to the derivatized aqueous extracts. They did not contain the complex peaks of a hydrocarbon oil. The sample which was glutaraldehyde hardened contained unique signals at retention times corresponding to scan numbers 294, 296 and 312 (Figures 2 and 3). The intensity of the peak at scan number 312 clearly increased in the spiking experiment where the protein sample was enriched with the oxime standard prepared under neutral pH from commercial glutaraldehyde (Figure 3). Note the additional peak introduced with the standard at scan number 173 which is a mono-derivatized glutaraldehyde, e.g., the molecular ion at m/z 295. The mono-derivative was formed because a relatively low excess of derivatizing agent was used.

The mass spectra of oxime derivatives detected in the glutaraldehyde hardened protein (e.g., scan numbers 294, 296 and 312 in Figure 2) were identical to the oxime derivative of the glutaraldehyde standard. All of these spectra contained characteristic features such as: a molecular ion at m/z 490, an M-197 loss (attributed to loss of $C_6F_5CH_2O$) and a strong fluorinated tropylium ion at m/z 181 (Figure 4). The peaks at scan numbers 294 and 296 are evidently due to other geometrical isomers, i.e., E/Z of glutaraldehyde oxime which may have formed under different pH conditions [5].

In one blank experiment, the derivatized pyrolysate of the heat hardened protein did not contain any peaks corresponding to derivatized glutaraldehyde.

In a separate blank experiment, glutaraldehyde was added to the unhardened protein at a concentration of approximately 10 ppm. This sample was pyrolysed and the subsequent analysis did show a peak corresponding to derivatized glutaraldehyde.

A GC equipped with a Mass Selective Detector was used to estimate the concentration of glutaraldehyde in the pyrolysate of the analysed protein. The relative intensities of both E/Z isomers were used to estimate the original glutaraldehyde concentration at approximately 1 ppm.

To the author's knowledge, this method represents the first example of pyrolysing a protein to detect glutaraldehyde.

The procedure developed was tested on several other proprietary protein samples. Although no glutaraldehyde was detected, one sample showed traces of glyoxal. According to industrial sources, some manufacturers use glyoxal as a hardening agent [2].

CONCLUSION

It appears that chemically hardened proteins can be distinguished from the non-chemically hardened proteins by detecting the residual hardening agents. The direct detection of hardening agents was not possible because of masking by complex signals produced by the hydrocarbon oils added to the hardened proteins. The problem with the oil-treated proteins was avoided by pyrolysing samples in a simple glassware device. The pyrolysate of the protein sample known to be glutaraldehyde hardened was derivatized with pentafluorobenzylhydroxylamine. The resulting oxime derivative was detected by GC-MS. The concentration of detected glutaraldehyde was estimated at approximately 1 ppm in the pyrolysate. The preliminary results also indicate that the method is potentially suitable for detecting other carbonyl containing hardening agents.

At the present time, this qualitative procedure is adequate for confirming that proteins have been hardened with a carbonyl

containing hardening agent such as glutaraldehyde. However, lack of detection of glutaraldehyde does not exclude the possibility that the protein may have been hardened by alternate means. This work could be expanded to a fully quantitative analytical method, subject to the availability of standards.

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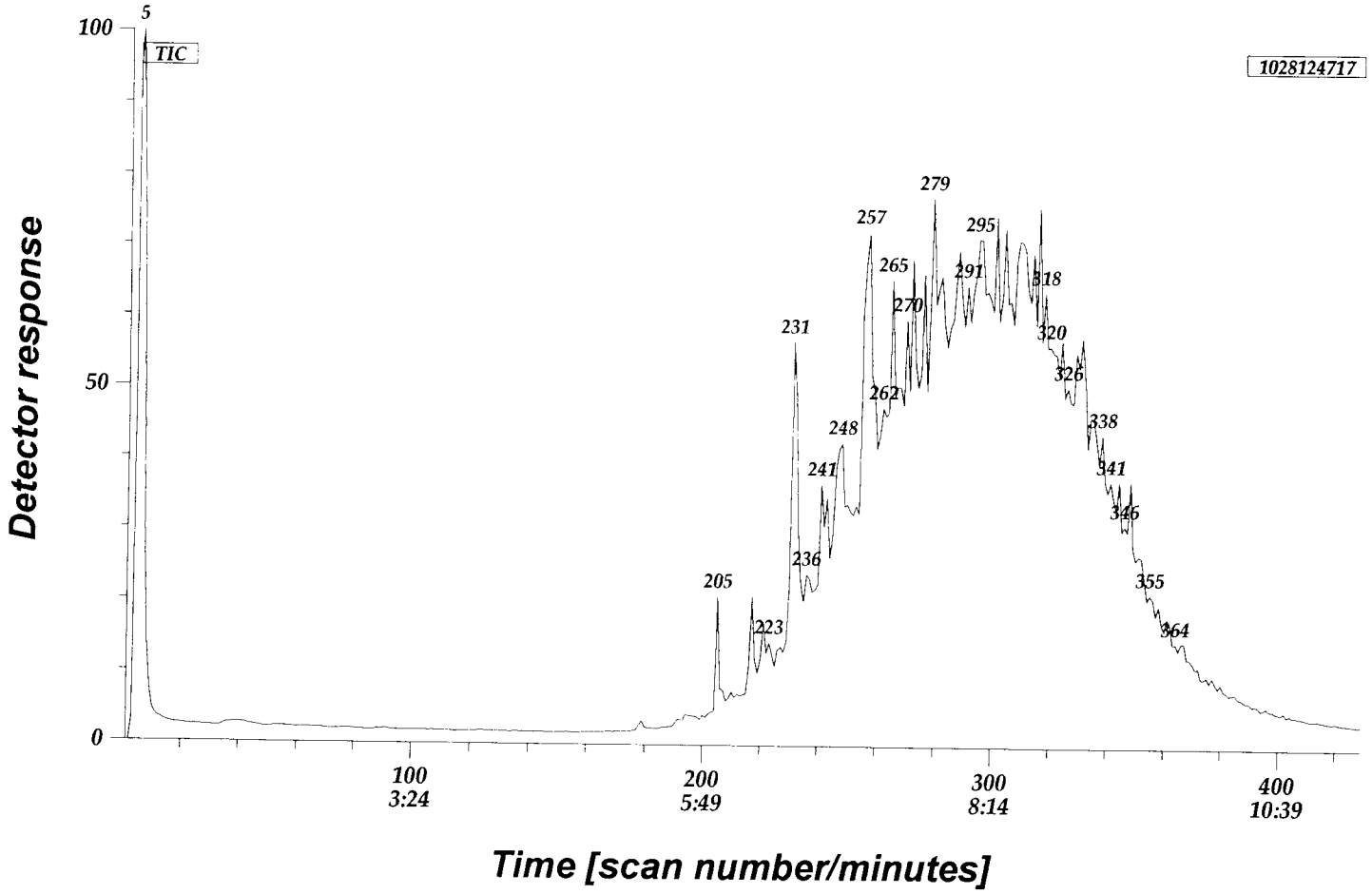
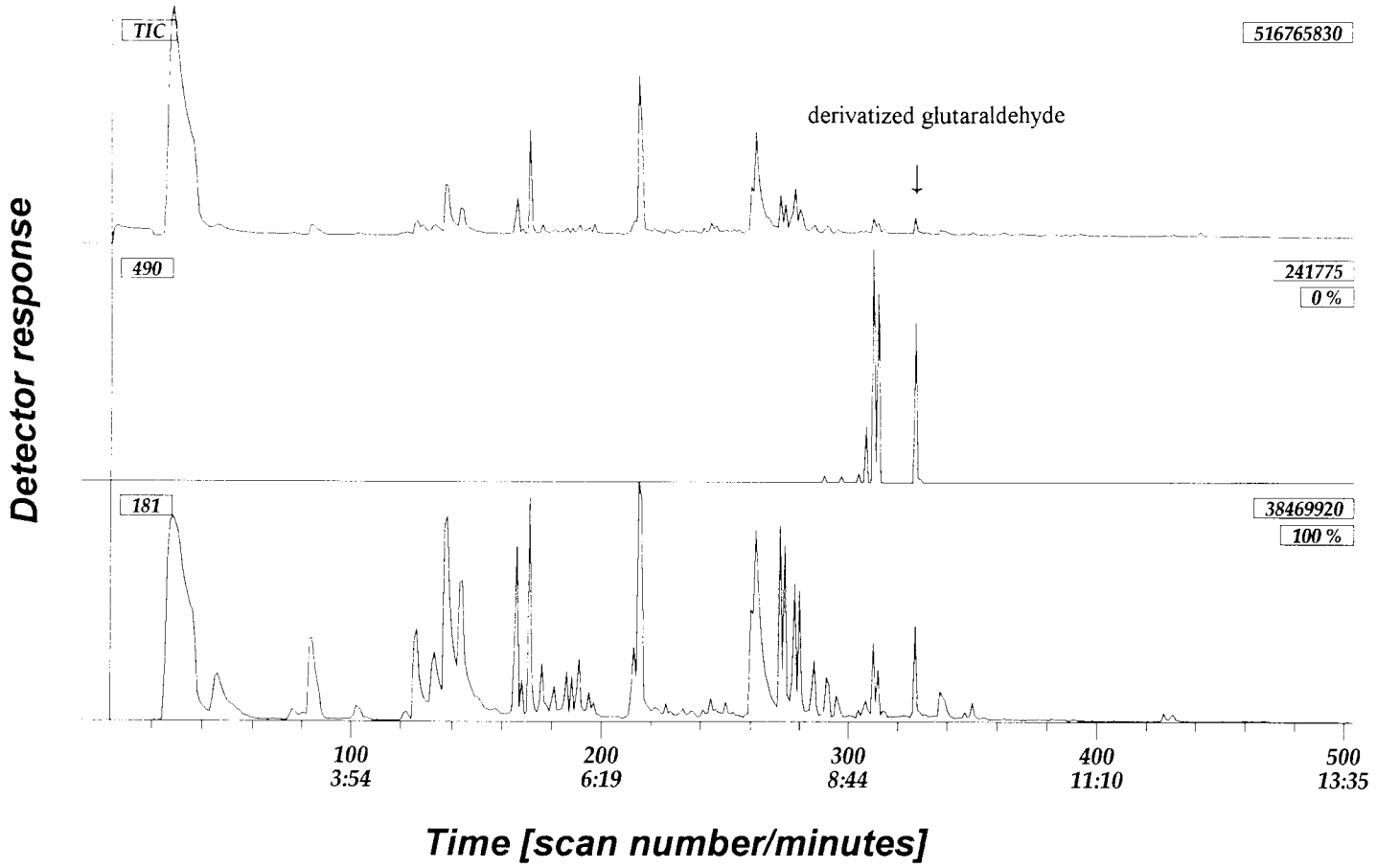
Figure 1



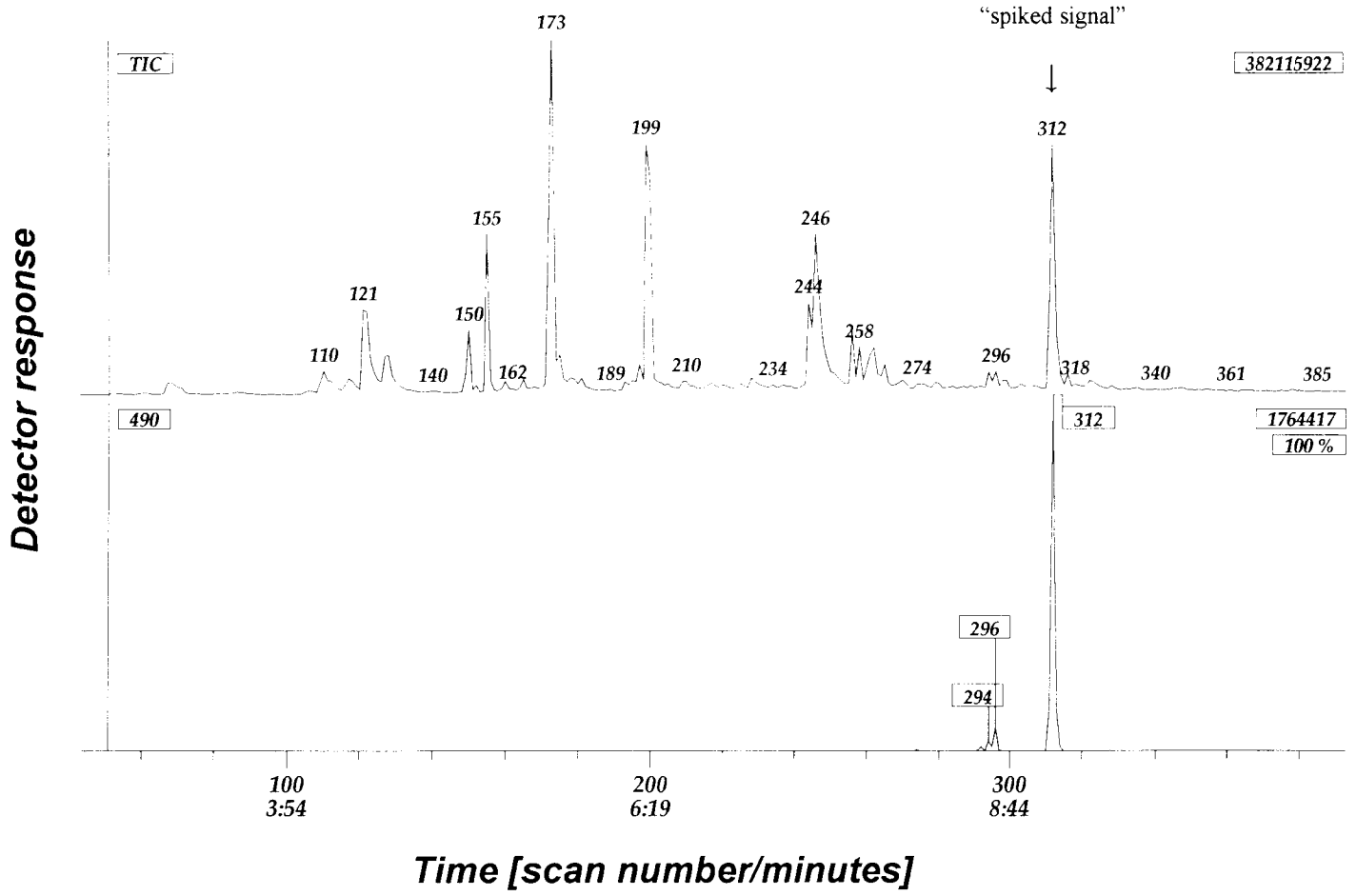
Chromatogram obtained from aqueous extract of glutaraldehyde hardened protein derivatised with PFBHA

Figure 2



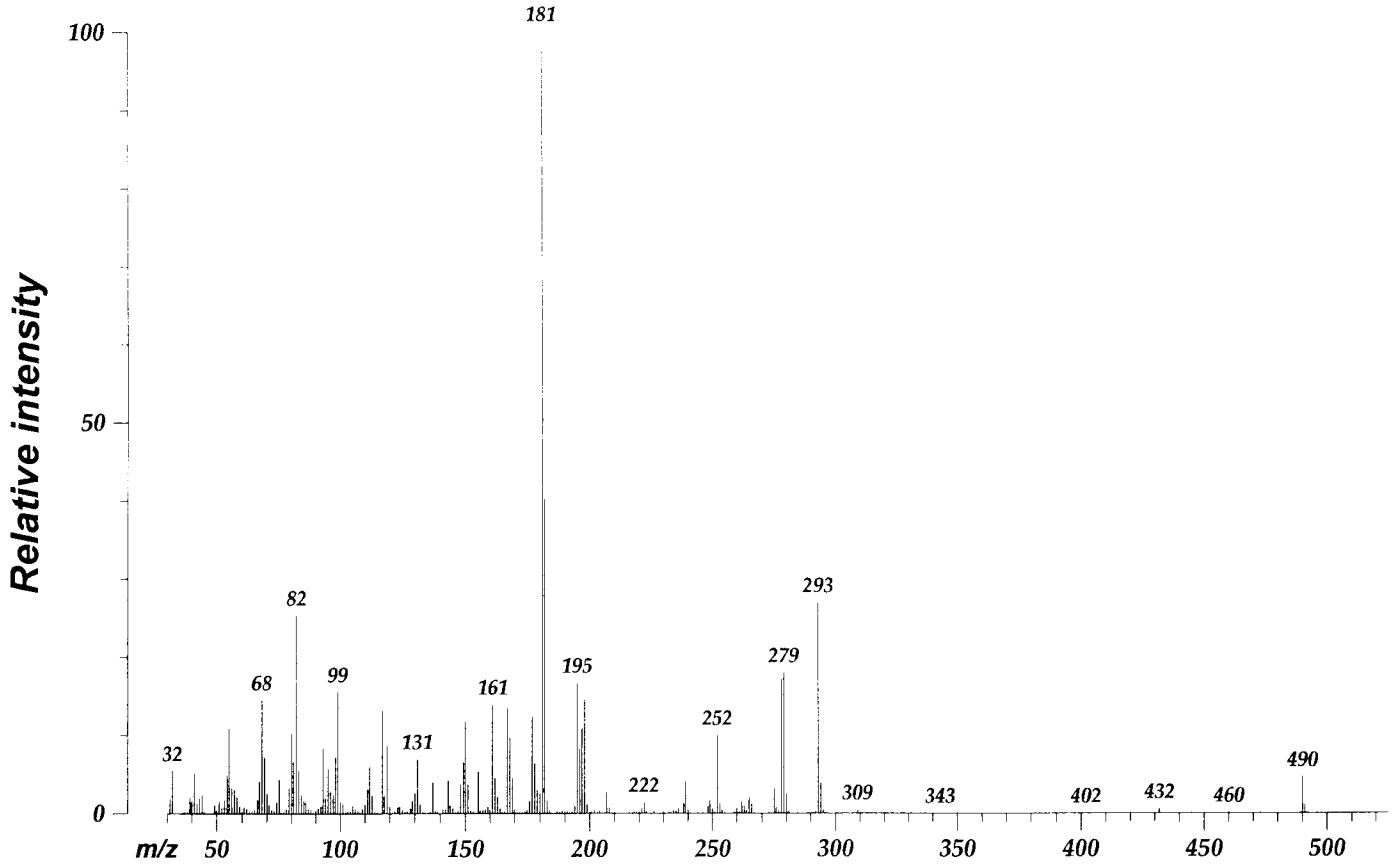
Chromatogram obtained from pyrolysate of glutaraldehyde hardened protein derivatised with PFBHA (the top trace represents a Total Ion Chromatogram; the middle trace shows only peaks with m/z of 490; the bottom trace is for peaks with m/z of 181)

Figure 3



Chromatogram obtained from pyrolysate of glutaraldehyde hardened protein spiked with commercial glutaraldehyde and derivatised with PFBHA(the top trace represents a Total Ion Chromatogram; the bottom trace shows only peaks with m/z of 490)

Figure 4



The Mass Spectrum of glutaraldehyde O-[(pentafluorophenyl)methyl] oxime